The Plant Cell, Vol. 9, 381–392, March 1997

Two cDNAs from Potato Are Able to Complement a Phosphate Uptake–Deficient Yeast Mutant: Identification of Phosphate Transporters from Higher Plants

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Acquisition as well as translocation of phosphate are essential processes for plant growth. In many plants, phosphate uptake by roots and distribution within the plant are presumed to occur via a phosphate/proton cotransport mechanism. Here, we describe the isolation of two cDNAs, StPT1 and StPT2, from potato (*Solanum tuberosum*) that show homology to the phosphate/proton cotransporter PHO84 from the yeast *Saccharomyces cerevisiae.* The predicted products of both cDNAs share 35% identity with the PHO84 sequence. The deduced structure of the encoded proteins revealed 12 membrane-spanning domains with a central hydrophilic region. The molecular mass was calculated to be 59 kD for the StPT1 protein and 58 kD for the StPT2 protein. When expressed in a PHO84-deficient yeast strain, MB192, both cDNAs complemented the mutant. Uptake of radioactive orthophosphate by the yeast mutant expressing either StPT1 or StPT2 was dependent on pH and reduced in the presence of uncouplers of oxidative phosphorylation, such as 2,4-dinitrophenol or carbonyl cyanide *m*-chlorophenylhydrazone. The K_m for Pi uptake of the StPT1 and StPT2 proteins was determined to be 280 and 130 μM, respectively. StPT1 is expressed in roots, tubers, and source leaves as well as in floral organs. Deprivation of nitrogen, phosphorus, potassium, and sulfur changed spatial expression as well as the expression level of StPT1. StPT2 expression was detected mainly in root organs when plants were deprived of Pi and to a lesser extent under sulfur deprivation conditions. No expression was found under optimized nutrition conditions or when other macronutrients were lacking.

INTRODUCTION

Phosphate is one of the key macronutrients required for plant growth and metabolism. It plays an important role in energy transfer through the formation of energy-rich phosphate esters and is also an essential component of macromolecules such as nucleotides, phospholipids, and sugar phosphates (Bieleski, 1973; Bieleski and Ferguson, 1983). Phosphate itself is involved in many processes in plants in that it is either the substrate or end product of a diverse range of metabolic reactions, and it is involved in the regulation of enzyme activity via phosphorylation (Marschner, 1995). Furthermore, the cytosolic Pi concentration has an impact on photosynthesis (Walker and Sivak, 1986; Usuda, 1995) and carbon partitioning (Rao et al., 1989).

Phosphate is tightly bound to inorganic or organic components of soils. Inorganic phosphate is absorbed to surface-active sesquioxides and oxihydrates of clay minerals or is precipitated as insoluble Ca^{2+} salts (Marschner, 1995). Organic phosphate is present as inositol phosphates, phospholipids, nucleic acids, and various other phosphate esters. These are derived from decaying organic matter and are often immobilized by sorption and fixation to soil colloids (Dalal, 1978). Hence, in most soils, mobilization and aquisition of phosphate often limit plant growth.

Acquisition of phosphate is probably a metabolically active process because simple mass flow caused by plant transpiration can satisfy only 2 to 3% of the total phosphate demand of a crop plant (Marschner, 1995). The creation of a phosphate depletion zone around the roots indicates an active absorption mechanism by the plant. In addition, plants accumulate higher concentrations of Pi than occurs in the soil. The Pi concentration of xylem sap exceeds that of an average soil solution by a factor of 100, and the concentration in vacuoles can be a factor of 10,000 times more than is the soil solution (Bieleski, 1973). Finally, a metabolically active Pi uptake process is required to overcome the electrochemical gradient arising from the negative membrane potential of the cell.

Several observations have led to the conclusion that both the uptake and internal transport processes within the plant

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occur via a proton symport mechanism. In pea root protoplasts, Pi influx was increased when the external pH was shifted to more acidic values. This uptake was sharply reduced by the application of uncouplers of oxidative phosphorylation such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Lefebvre and Clarkson, 1984), indicating that the presence of a proton motive force is necessary to drive Pi uptake. Acidification of the root cytoplasm in root cells of Limnobium during Pi uptake was demonstrated with pH electrode measurements (Ullrich and Novacky, 1990). Similar results were found with suspension-cultured cells of *Catharanthus roseus* with the help of pH-dependent fluorescent dyes (Sakano et al., 1992). Furthermore, transport within the plant and deposition in storage compartments seem to be energy-dependent processes (Mimura, 1995). A proton symport is active in leaves, as demonstrated by phosphorus-32 feeding experiments with detached barley leaves and phosphorus-32 uptake studies with barley mesophyll protoplasts (Mimura et al., 1990, 1992). The stoichiometry of Pi/proton cotransport is still being debated. A ratio of 1:1 has been reported for maize and white clover (Sentenac and Grignon, 1985; Dunlop, 1989). However, a stoichiometry of more than one proton per Pi has been suggested for *Lemna gibba* (Ullrich-Eberius et al., 1984) and *C. roseus* (Sakano, 1990).

A remarkable feature of phosphate uptake in higher plants is the enhanced rate of Pi absorption of Pi-deprived plants when the nutrient is replenished (Clarkson and Lüttge, 1991). The increased uptake may be due to activation of existing transporters or to the synthesis of other transporters (Dunlop and Gardiner, 1993). The increased uptake capacity caused by Pi deprivation is highly specific for phosphate because no increased uptake of other anions has been observed (Lee, 1982).

cDNA or genomic clones encoding phosphate transporters from eukaryotes have been isolated from mammalian species (e.g., Na^+/phosphate cotransporter encoding cDNA BNP1 from rat, Binhui et al., 1994), from *Neurospora crassa* (e.g., genomic clone *pho-4*$^+$, Mann et al., 1989; and genomic clone *pho-5*$^+$, Versaw, 1995), and from the mycorrhizal fungus *Glomus versiforme* (H^+/phosphate cotransporter encoding cDNA GvPT, Harrison and van Buuren, 1995). Two Pi transport systems have been described to date in the eukaryotic organism *Saccharomyces cerevisiae*, one displaying a low K_m value (8.2 μM) and another displaying a high K_m value (770 μM). The low K_m system is encoded by the *PHO84* gene (Bun-ya et al., 1991). The PHO84 protein has been shown to transport phosphate in a pH-dependent manner (Bun-ya et al., 1991; Berhe et al., 1995). No full-length cDNA that encodes a proton/phosphate cotransporter from higher plants has as yet been described. In this study, we report the cloning and functional characterization of two cDNAs from potato (*Solanum tuberosum*) and the expression of both cDNAs in the yeast *S. cerevisiae*.

RESULTS

Deduced Peptide Sequence of Two Root-Derived cDNAs Displays a Six-Loop-Six Protein Structure Typical of Membrane Proteins

Expressed sequence tag clone 134M11T7 (Arabidopsis Biological Resource Center, Ohio State University, Columbus; GenBank and EMBL accession number T46507) was used as a probe to screen a root cDNA library. The roots were derived from potato plants deprived of all nutrients for 3 days to enhance ion uptake capacity. After plaque purification and in vivo excision, two clones (p12 and p21) were isolated.

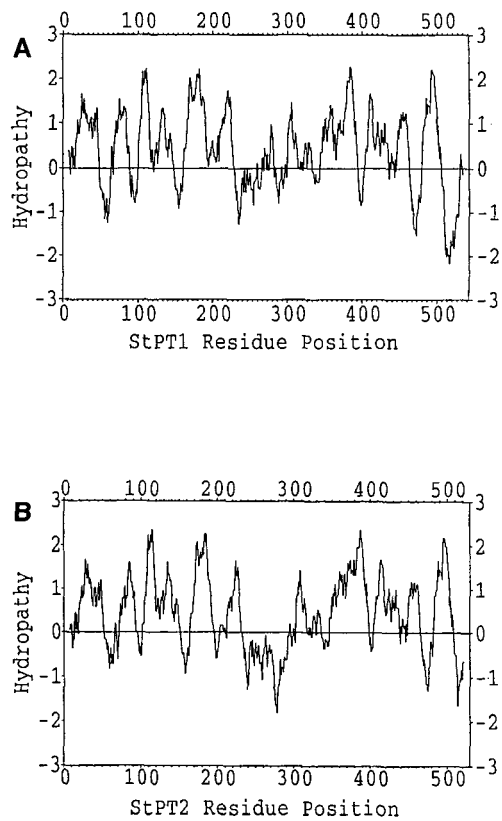

Figure 1. Hydropathy Plot Calculated from the Deduced Amino Acid Sequence of StPT1 and StPT2.

The calculation was done according to the algorithm of Kyte and Doolittle (1982) with a window size of 15 amino acids. Positive index values refer to hydrophobic regions of the peptide. The hydropathy plot of StPT1 and StPT2 proteins displays a six-loop-six structure of the six hydrophobic N- and C-terminal domains that are separated by a central hydrophilic region.

(A) StPT1 protein.

(B) StPT2 protein.

The cDNA insert of p12, designated StPT1, is 1812 bp long. The longest open reading frame (ORF) starts with an ATG start codon at position 101, and an in-frame TAG stop codon is present at position 1721, yielding an ORF of 1620 bp. The cDNA insert of p21, designated StPT2, is 1956 bp long, with an ATG start codon at position 139 and an in-frame TGA stop codon at position 1820, yielding an ORF of 1681 bp. Neither a poly(A) nor the putative polyadenylation signal AATAAA (Joshi, 1987) was found in either cDNA. The accession numbers of StPT1 and StPT2 in the EMBL data base are X98890 and X98891, respectively.

StPT1 and StPT2 encode peptides of 540 and 527 amino acids, respectively. Hydropathy plots of the predicted proteins show the presence of 12 hydrophobic segments, 15 to 20 amino acids in length, that have an average hydropathy index of >1.3 (Kyte and Doolittle, 1982). A central hydrophilic domain separates six N-terminal domains from six C-terminal segments (Figures 1A and 1B). Hydrophobic domains are thought to span the membrane, whereas the hydrophilic domain faces the cytoplasm, as do the N and C termini of the peptide. This secondary structure, termed a six-loop-six structure, occurs in PHO84 as well as in other membrane transporters such as amino acid and sugar transporters from prokaryotes and eukaryotes (Reizer et al., 1994). The relative molecular masses were calculated to be 59 kD for the StPT1 protein and 58 kD for the StPT2 protein. Putative phosphorylation sites are present as protein kinase C phosphorylation sites at positions 238 to 240 displaying the [ST]-x-[RK] consensus motif and at positions 510 to 513 as casein kinase II phosphorylation sites after the [ST]-x(2)-[DE] consensus motif (numbers refer to the amino acid sequence of the StPT1 protein as shown in Figure 2). An N-glycosylation site is present at positions 422 to 425 displaying the consensus motif N-{P}-[ST]-{P}. (Consensus sequences are given according to the PROSITE dictionary of protein sites and patterns; see Methods.) Only these sites that are also present in PHO84 and phosphate transporters from other eukaryotes are mentioned.

Structure of the Predicted StPT1 and StPT2 Proteins Displays Significant Homology to Other H^+/Pi Cotransporters but No Homology to Na^+/Pi Cotransporters

There is 78.5% identity between the amino acid sequences of the polypeptides encoded by StPT1 and StPT2. Regions of nonidentity may indicate different physiological functions or transport characteristics. StPT1 and StPT2 proteins have the highest homology with the GvPT protein from the mycorrhiza *G. versiforme* (Harrison and van Buuren, 1995), displaying 41.7 and 41.8% amino acid sequence identity, respectively. They share 34.9 and 36% amino acid identity with PHO84 from *S. cerevisiae* (Bun-ya et al., 1991) and show almost the same identity to PHO5 from *N. crassa* (Versaw, 1995). A second class of phosphate transporters uses sodium ions instead of protons and is widespread in animal cells (e.g., the BNP1-encoded protein from rat; Binhui et al., 1994). StPT1 and StPT2 proteins show no significant homology to members of this class of phosphate transporters when amino acid data are compared. Nor was there significant homology to the recently described sulfate transporter family (Smith et al., 1995) or to cation channels. Table 1 summarizes identity data calculated using the BESTFIT algorithm software of the Genetics Computer Group (Madison, WI).

Expression of StPT1 and StPT2 Leads to Complementation of a Phosphate Uptake–Deficient Yeast Strain

The yeast phosphate uptake mutant MB192 (BunYa et al., 1991) was chosen to obtain further evidence for the proposed function of StPT1 and StPT2 proteins as phosphate transporters. In MB192, the *PHO84* gene was disrupted by insertion of an *HIS3* DNA fragment and so lacks high-affinity phosphate transport activity. This mutant exhibits a reduced phosphate acquisition capacity and constitutively expresses a Pi-repressible acid phosphatase (rAPase; EC 3.1.3.2) even at phosphate concentrations in the medium that otherwise repress rAPase synthesis in the wild-type yeast. Expression of rAPase is under the control of the same PHO regulatory system that regulates *PHO84* expression. With the help of this system, Pi starvation derepresses the *PHO84* ORF and activates rAPase synthesis. Pi concentration within the yeast cell is strongly influenced by the activity of PHO84 itself (Bun-ya et al., 1991).

StPT1 and StPT2 were subcloned into the NotI site of the yeast expression vector p112A1NE (Riesmeier et al., 1992). The polymerase chain reaction product of the genomic *PHO84* gene in the same yeast expression vector was used as a control. Transformants of MB192 were named YStPT1, YStPT2, and YPHO84, respectively. Figures 3A and 3B show the MB192 mutant and the YStPT1, YStPT2, and YPHO84 transformants on phosphatase staining medium. This medium reveals rAPase activity of yeast colonies by staining them red. The mutant yeast stained red (Figures 3A, middle, and 3B, right), but the YStPT1 (Figure 3A, left) and YStPT2 (Figure 3A, right) transformants remained pale, as did the control strain YPHO84 (Figure 3B, left). Transformants harboring the p112A1NE expression plasmid without any insert also stained red (data not shown).

Because mutant MB192 is devoid of an important phosphate transport system, its growth is retarded compared with that of the wild type. To compare growth characteristics of the mutant yeast and the transformants, liquid medium containing low phosphate (140 μM) was inoculated with YStPT1, YStPT2, YPHO84, or MB192 transformed with the empty expression plasmid p112A1NE (termed MB192(pl)),

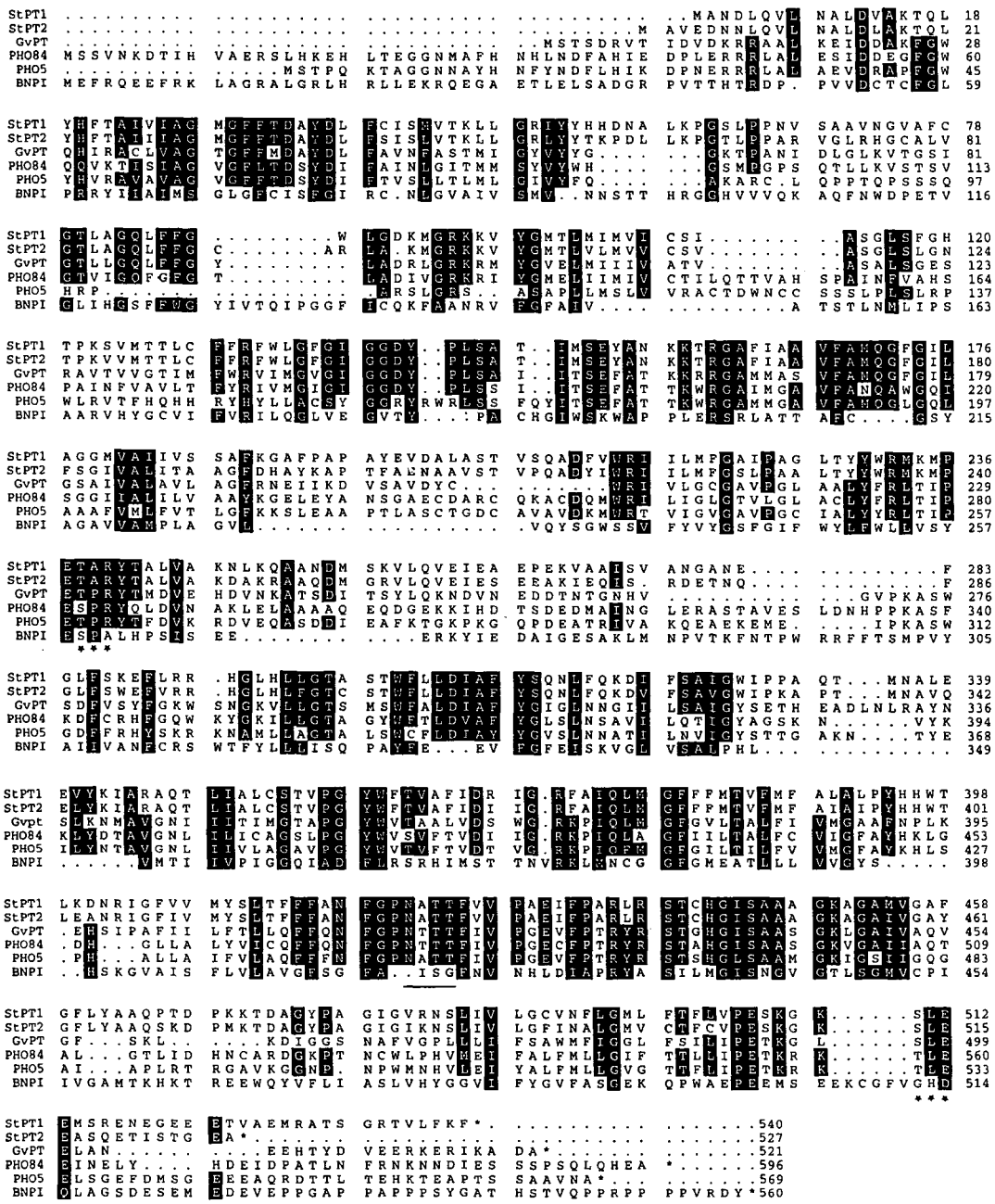

Figure 2. Sequence Alignment of StPT1 and StPT2 Protein Amino Acid Sequences with Known Phosphate/Proton Symporters of Other Organisms.

Shown is the alignment of the predicted amino acid sequence of StPT1 and StPT2 proteins with amino acid sequences of PHO84 from *S. cerevisiae* (Bun-ya et al., 1991), the GvPT protein from the mycorrhiza *G. versiforme* (Harrison and van Buuren, 1995), Pho-5 of *N. crassa* (Versaw, 1995), and the BNP1 protein from rat (Binhui et al., 1994). Black boxes indicate identical amino acid residues. Asterisks within the sequence indicate a stop codon in the nucleotide sequence. Dots mark the spacing in the amino acid sequence that was done for proper alignment. Asterisks below the sequence indicate putative phosphorylation sites, and the underlined sequence is a consensus site for N-glycosylation.

Table 1. Percentage of Identity of the Deduced Amino Acid Sequences of StPT1 and StPT2 Proteins and Other Phosphate Transporter Proteins

Phosphate Transporter	StPT1 Identity (%)	StPT2 Identity (%)
StPT1 protein (potato)	100	78.5
StPT2 protein (potato)	78.5	100
GvPT protein (*G. versiforme*)	41.7	41.8
PHO84 (yeast)	34.9	36.0
PHO-5 (*N. crassa*)	33.2	35.8
BNP1 protein (rat)	19.6	18.4

respectively (Figure 4A). Growth was monitored by measuring the optical density at 660 nm. Transformants with StPT1 and StPT2 did not regain the full wild-type growth characteristics of YPHO84. However, expression of both cDNAs enabled the mutant to grow significantly faster than did the mutant strain harboring the empty expression plasmid.

Expression of StPT1 and StPT2 in Yeast Leads to an Increased Uptake of Inorganic Phosphate

To obtain additional support for the biochemical function of StPT1 and StPT2 proteins, direct uptake of phosphate by yeast transformants was analyzed. Figure 4B shows that expression of StPT1 and StPT2 leads to a significantly higher phosphate uptake than either the mutant yeast or the mutant yeast bearing p112A1NE. Pi uptake by the StPT1 and StPT2 transformants was approximately one-third that of the mutant transformed with the endogenous *PHO84* gene.

The influence of pH on the uptake of radioactive orthophosphate by the YStPT1 and YStPT2 transformants was tested. Uptake was increased as the pH of the medium was reduced from 7.5 to 4.5 (Figure 5). To investigate the influence of a proton motive force on phosphate transport activity, the uncouplers 2,4-dinitrophenol (2,4-DNP) or CCCP were applied. A concentration of 100 μM 2,4-DNP reduced phosphate uptake to 58% (YStPT1) and 55% (YStPT2) compared with a 100% uptake of controls in which no inhibitors were given. CCCP (10 μM) reduced uptake to 75% (YStPT1) and 58% (YStPT2). Inhibitors were added to activated cells 30 sec before the addition of radioactive orthophosphate. This corroborates the pH dependence of StPT1 and StPT2 protein-mediated phosphate transport, because these compounds destroy the pH gradient across the yeast cell membrane, which is necessary for proper function of proton/solute symporters. The transport of Pi into cells of YStPT1 and YStPT2 follows Michaelis-Menten kinetics with an apparent K_m for Pi of 280 and 130 μM for the StPT1 and StPT2 proteins, respectively (Figures 6A and 6B).

StPT1 and StPT2 Show Different RNA Expression Patterns

The expression of StPT1 and StPT2 in plants grown under optimal or limiting nutrition conditions was analyzed by RNA gel blot analysis. High-stringency washings were used to prevent cross-hybridization with either cDNA probe. RNA analysis from different tissues of soil-grown potato plants, using StPT1 as a probe, produced a hybridization band at ~1.8 kb, which is in good agreement with the length of the cDNA. In these plants, StPT1 is expressed in roots, mature leaves, and flower buds and at a lower level in growing tubers, sprouting tubers, and flowers. Expression could not be detected in stem or young leaves. Enhanced expression was found in roots derived from plants grown for 3 days on limiting nutrients (Figure 7A).

StPT2 could not be detected by RNA gel blot analysis in tissue derived from soil-grown plants, but it was strongly expressed in nutrient-deprived roots (Figure 7B). The hybridization at 1.8 kb again matched the length expected from the cDNA. These results indicate that expression of the StPT1- and StPT2-encoding genes responds to changing nutrition conditions.

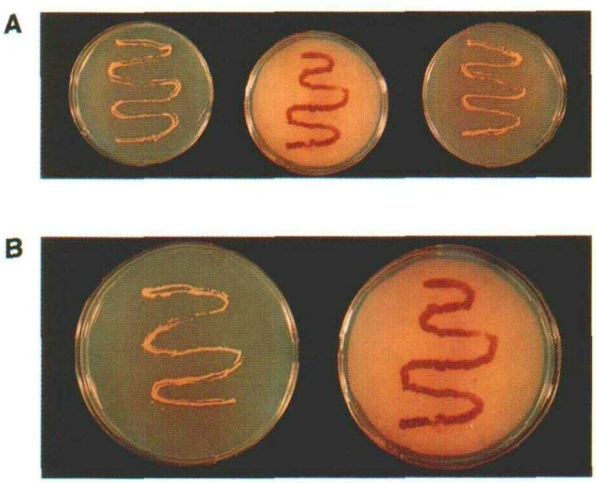

Figure 3. Staining Test for Acid Phosphatase Activity.

(A) An acid phosphatase activity test was conducted with yeast mutant strain MB192 (middle) and transformants YStPT1 (left) and YStPT2 (right). The latter contain StPT1 and StPT2 cDNAs subcloned in the yeast expression vector p112A1NE. Phosphatase activity was detected by staining according to Bun-ya et al. (1991). Whereas mutant colonies stain red, the transformants remain pale.

(B) As a control, the *PHO84* polymerase chain reaction product in p112A1NE was transformed in MB192, yielding transformed strain YPHO84. YPHO84 (left) reveals the same staining phenotype as YStPT1 and YStPT2 and differs from the phenotype of MB192 (right). The phenotype of MB192 transformed with expression plasmid p112A1NE containing no insert (called MB192(pl)) was the same as without transformation (data not shown).

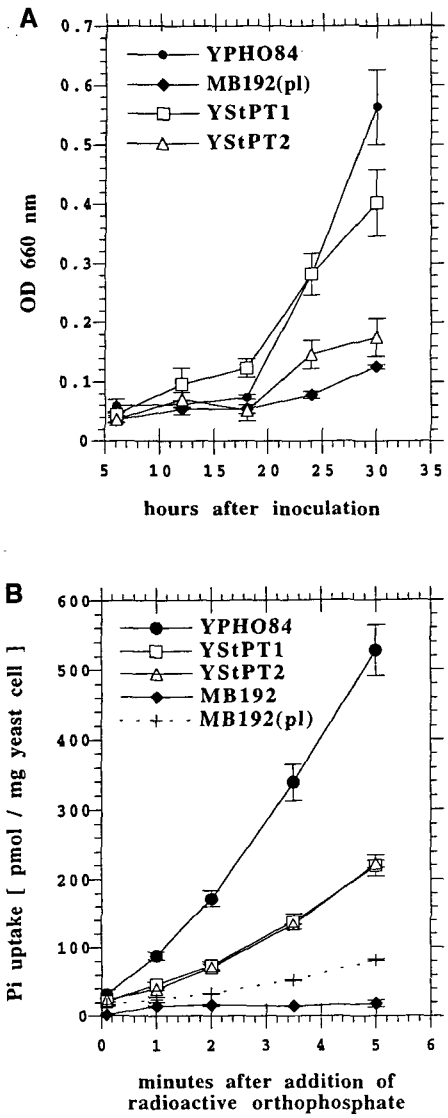

Figure 4. Phenotype of YStPT1 Carrying Expression Plasmid p112A1NE Containing the Phosphate Carrier–Encoding cDNA StPT1 and of YStPT2 Carrying the Same Plasmid Containing StPT2.

(A) Growth of YStPT1 and YStPT2 compared with mutant strain MB192(pl) carrying p112A1NE without an insert and YPHO84 carrying p112A1NE containing the *PHO84*-encoding polymerase chain reaction product.

(B) Uptake of radioactive orthophosphate by YStPT1, YStPT2, YPHO84, MB192(pl) containing p112A1NE without an insert, and untransformed MB192. Cells were grown in YNB medium at a phosphate concentration of only 140 μM KH_2PO_4 and were preincubated 5 min before uptake with 10 μM glucose.

To obtain further insight into regulation of these genes, we grew plants in quartz sand cultures on 0.5 × modified Hoagland's medium lacking phosphorus, sulfur, nitrogen, or potassium for 2 weeks after a period of optimized nutrient conditions. Total RNA was prepared from young and mature leaves, stems, roots, stolons, and developing tubers, and blots were probed with StPT1 or StPT2. StPT1 showed expression in all tissues under all conditions applied (Figure 8A). Faint expression was also detected in stem and sink leaves in unmodified Hoagland's medium (data not shown). StPT2 was highly expressed in roots of plants starved of Pi (Figure 8B). It was also expressed in other nonaerial parts (stolons and developing tubers) of Pi-deprived plants. Weak expression of StPT2 was also detected in the roots of plants deprived of sulfate (Figures 9A and 9B). StPT2 expression was not detected in aerial parts of phosphorus-deprived plants or in any tissues of plants deprived of potassium or nitrogen.

DISCUSSION

Physiological observations have led to the assumption that a phosphate/proton symport mechanism is responsible for the energy-dependent transport of phosphate by plant cells. However, the presence of genes encoding plant transmembrane proteins with characteristics of secondary active transporters has not yet been demonstrated at the molecular level.

Here, we describe two full-length cDNA clones, StPT1 and StPT2, from a potato root library, the deduced amino acid sequences of which display structural similarity to eukaryotic phosphate transporters. The polypeptides encoded by these genes have sequence homology to polypeptides en-

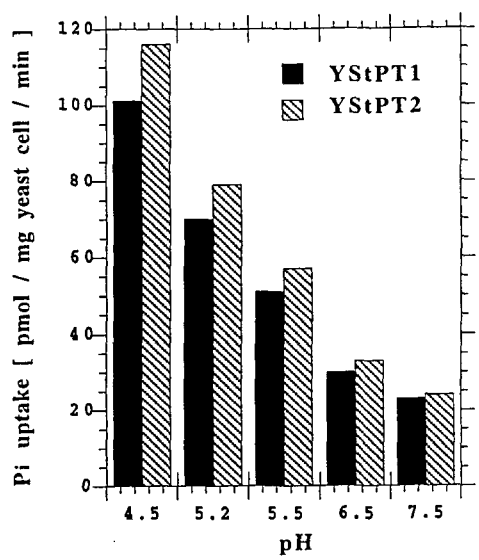

Figure 5. Uptake Rate of Radioactive Orthophosphate by Strain YStPT1 and YStPT2 at Different pH Values of the Medium.

pH values are measured values. Mes buffer was used to buffer at a pH range of 5.5 to 7.0.

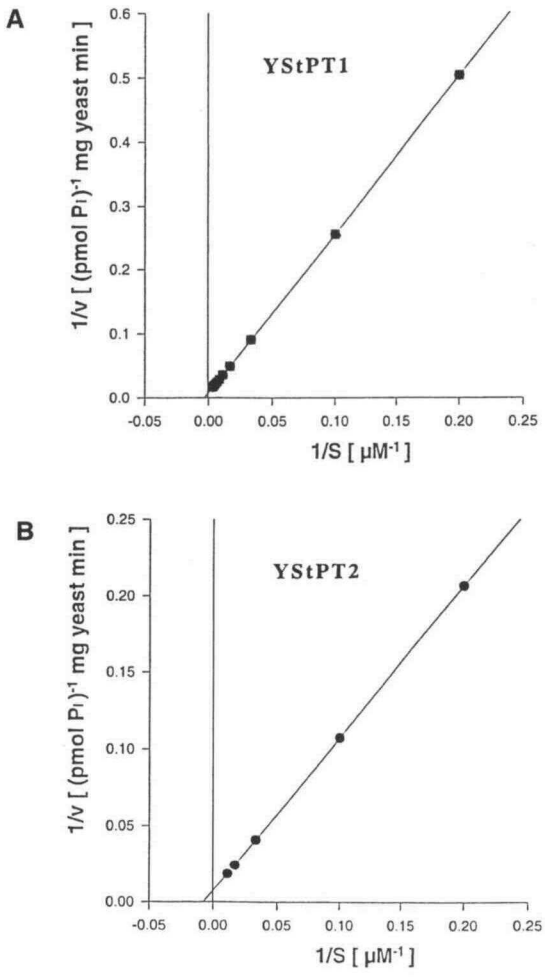

Figure 6. Velocity of Pi Transport by YStPT1 and YStPT2 as a Function of Pi Concentration.

Shown is a Lineweaver-Burk plot of phosphate incorporation rate versus external phosphate concentration that was used to estimate K_m values. Uptake was determined per milligram of yeast cells. 1/v is the reciprocal of the uptake velocity, which is defined as the uptake of picomoles of Pi per milligram of yeast per minute. 1/S is the reciprocal of the substrate concentration of the uptake medium, which is given in µM Pi.

(A) YStPT1.

(B) YStPT2.

coded by the cDNA from the mycorrhizal fungus *G. versiforme* (GvPT) and by genes from the yeast *S. cerevisiae* (*PHO84*) and the fungus *N. crassa* (*pho-5*$^+$), which have been characterized as phosphate/proton symporters (Bun-ya et al., 1991; Berhe et al., 1995; Harrison and van Buuren, 1995; Versaw, 1995). No significant homology was found to the sulfate transporter family (Smith et al., 1995) or to cDNAs encoding mammalian Na^+/phosphate cotransporters (e.g., BNP1; Binhui et al., 1994).

The deduced amino acid sequence of StPT1 and StPT2 suggests a structure containing 12 membrane-spanning domains separated by a central hydrophilic loop (Reizer et al., 1994). This structure has also been identified in other plant membrane transporters, such as the sucrose transporter of potato (Riesmeier et al., 1992). Furthermore, the amino acid sequences of StPT1 and StPT2 proteins show putative phosphorylation sites and a glycosylation site. The importance of these sites is not known, but these motifs are also present in the fungal phosphate/proton symporters that have been described previously.

The orthophosphate transport capacity of StPT1- and StPT2-encoded proteins was determined by using heterologous expression of these cDNAs in yeast. Performing direct uptake assays for radioactive orthophosphate with the high-affinity phosphate transporter yeast mutant MB192 showed an enhanced ability of the StPT1 and StPT2 transformants to regain phosphate transport capacity. Transformants in which StPT1 and StPT2 were expressed also grew faster on low-phosphate medium than did the mutant MB192. Because of the restored phosphate uptake capacity when expressing either of the cDNAs in MB192, rAPase was no longer secreted. This enzyme was secreted constitutively in the mutant, because its synthesis is induced by a low intra-cellular Pi concentration caused by the loss of PHO84 function. In StPT1- and StPT2-transformed cells, however, phosphate accumulated at concentrations high enough to repress rAPase formation and produced the same

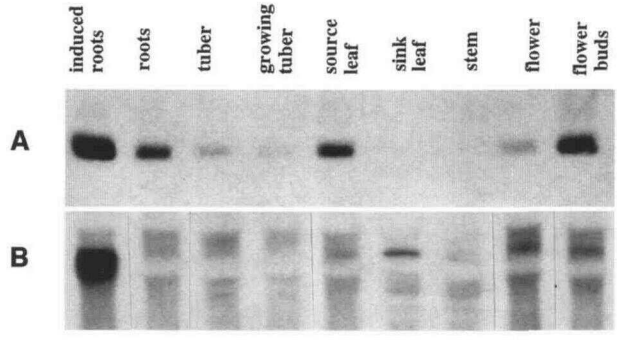

Figure 7. Gel Blot Analysis of Potato RNA Derived from Different Tissues.

(A) Thirty micrograms of total RNA in each lane was hybridized with StPT1.

(B) The same amount of RNA as given in **(A)** was hybridized with the StPT2 full-length cDNA.

Hybridization shows the occurrence of a single RNA transcript at a length of 1.8 kb. Roots derived from nutrient-starved plants are termed induced roots.

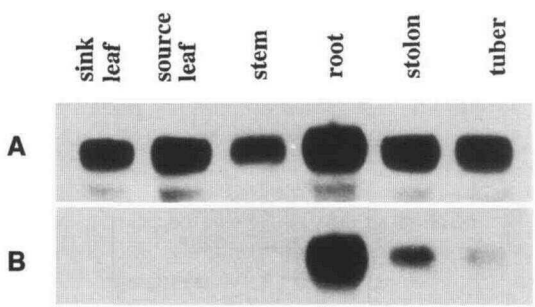

Figure 8. Gel Blot Analysis of RNA from Potato Derived from Roots of Phosphate-Deprived Plants.

(A) Thirty micrograms of total RNA hybridized with an StPT1-specific probe revealing constitutive expression of an StPT1-encoding mRNA. The same expression pattern was also seen with sulfur-, potassium-, and nitrogen-deprived plants (data not shown). Plants nourished on full Hoagland's medium also showed faint expression of StPT1 in stem and sink leaves.

(B) Hybridization pattern of StPT2 using the same blot as shown in **(A)**. No hybridization was detected with RNA from the above-mentioned cultures, with the exception of sulfur-deprived plants, which showed weak hybridization with StPT2 to root-specific RNA (see Figure 9). In all cases, the hybridizing RNA species was ~1.8 kb.

phenotype as that resulting from a complementation by the yeast *PHO84* gene.

The transport of phosphate across plant membranes driven by a proton/phosphate symporter mechanism is likely to be pH dependent. The observed increase in Pi uptake rates when pH decreased is consistent with the operation of a proton/Pi symporter. The high phosphate uptake rate at pH 4.5 could be due to the pH optimum of the protein itself or might indeed reflect that the transport mechanism is a phosphate/proton symport. However, the reduced uptake in the presence of uncouplers of pH gradients across membranes, such as 2,4-DNP and CCCP, favors the latter interpretation. This view is supported by the finding that preincubation with glucose before the uptake experiment with radioactive phosphate strongly enhances the uptake capacity of the transformants (data not shown). This effect could be caused by an enhanced proton extrusion that may result from preincubation with glucose. Hence, similarities to the six-loop-six topology of plant membrane transporters, homology data with established proton/Pi cotransporter, and functional analysis in the yeast mutant MB192 indicate that StPT1 and StPT2 probably encode phosphate transporters.

Phosphate concentrations in soil solution range from 0.5 to 2 μM, depending on the soil composition (Bieleski and Ferguson, 1983). Therefore, a high-affinity phosphate uptake system in the plant root is likely to operate with a K_m value for Pi uptake that covers this concentration range. Cogliatti and Clarkson (1983) measured K_m values of Pi transport for roots of Pi-sufficient and Pi-deprived potato plants of 21 and 2 μM, respectively. Different K_m values for Pi uptake into roots and protoplasts have been reported from other plant species, ranging from 2.5 to 74 μM (Shimogawara and Usuda, 1995). However, the K_m values of Pi uptake of 280 μM for the StPT1 protein and 130 μM for the StPT2 protein, as determined in yeast, diverge significantly from K_m values of these experiments. Obviously, high-affinity Pi transport is not obtained when StPT1 and StPT2 are expressed in the heterologous yeast background. The reasons for this may be threefold.

First, StPT1 and StPT2 proteins may indeed be low-affinity phosphate transporters. These transporters could be involved in internal phosphorus flow between different plant tissue. Such fluxes have been reported for different plant species under Pi deprivation (Smith et al., 1990; Mimura et al., 1996).

Second, the putative phosphorylation sites in StPT1 and StPT2 proteins may indicate that post-translational modification is required for proper protein function. Furthermore, the transporter itself may be composed of different subunits, as was shown for nitrate transport in *Chlamydomonas reinhardtii* (Quesada et al., 1994), in which at least two components were required to fully complement nitrate transport mutants. The involvement of additional components, such as an extracellular, cell wall–bound Pi binding protein in high-affinity Pi transport by plants, has been proposed. Maas et al. (1979) demonstrated in barley roots that osmotic shock caused a decrease in phosphate uptake together with a release of proteins into the medium. Jeanjean (1975) and co-workers (1981) found that extracellular Pi binding proteins in *Chlorella pyrenoidosa* and *Candida tropicalis* were dislodged by osmotic shock, leaving Pi transport with a significantly higher K_m value. However, Lefebvre and Clarkson

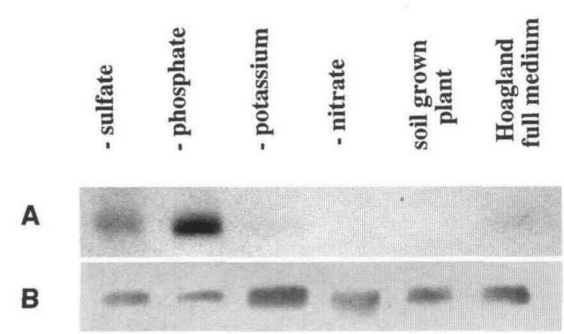

Figure 9. RNA Gel Blot Analysis of Root-Specific RNA Derived from Plants Starved for Phosphorus, Sulfur, Nitrogen, and Potassium as Well as from Plants Grown under Optimal Nutrient Conditions.

(A) Hybridization of StPT2 with 30 μg of total RNA shows the presence of an StPT2-specific mRNA with RNA from Pi-deprived plants. The presence of StPT2-specific mRNA was also detected to a lesser extent in sulfur-starved plants.

(B) To indicate the total amount of RNA present, hybridization was performed with 25S rRNA as a control.

(1986) showed that in pea root cells, the high affinity for phosphate influx was not significantly altered by osmotic shock, plasmolysis, or preparation of free protoplasts, indicating the independence of phosphate uptake from extracellular compounds.

Third, it has been shown that in yeast, high-affinity Pi transport requires collaboration of several different proteins to form a functional unit. The *PHO84* gene product seems to interact with PHO86, PHO87, Gtr1p, and PHO88 (Bun-ya et al., 1992, 1996; Yompakdee et al., 1996). Heterologous expression of plant phosphate transporters may interfere with the proper protein interaction to result in an only low-affinity Pi transport. The presence of a multicomponent system for high-affinity Pi uptake in yeast finally raises the question of whether such a system is also present in plants.

To investigate spatial expression and regulation of StPT1 and StPT2 in plants, we performed RNA gel blot analysis under different nutrient conditions. The expression of StPT1 in tubers, roots, leaves, and flowers indicates that StPT1 is a more ubiquitious transport protein, with Pi translocating functions within the whole plant. The transcription of StPT1 is enhanced under nutrient deprivation in roots compared with the expression of soil-grown plants. This may reflect an increase in the synthesis of carrier proteins, which has been proposed by several authors as a response to nutrient starvation (Clarkson and Lüttge, 1991; Dunlop and Gardiner, 1993).

In contrast to StPT1, StPT2 is expressed mainly in roots and to a minor extent in other nonaerial organs such as tubers and stolons. Expression is enhanced under phosphate deprivation. Sulfate limitation also seems to enhance expression in roots somewhat but does not increase expression in tubers or stolons. The expression pattern of StPT2 suggests that the plant responds to phosphate limitation by inducing the expression of an additional phosphate transporter gene. This result supports observations with white clover, showing that an enhanced rate of phosphate uptake due to phosphate starvation was accompanied by a change in proton/phosphate stoichiometry and by altered sensitivity of phosphate transport to the sulfhydryl-binding reagent *N*-ethylmaleimide (Dunlop and Gardiner, 1993). Cogliatti and Clarkson (1983) found that under Pi deprivation conditions, there was a 10-fold decrease in the K_m value for Pi uptake into pea roots when deprived of phosphate. These findings can be interpreted as derepression of a different phosphate transporter that, in addition to the constitutive one, mediates phosphate transport under conditions of phosphate deprivation. These physiological changes may reflect induced transcription of StPT2 at the molecular level.

Interestingly, a new phosphate-transporting activity, indicated by StPT2 expression, can be induced by only 3 days of deprivation conditions after several weeks at optimized nutrition conditions. Katz et al. (1986) suggested that a long period of phosphorus deprivation is needed for changes in transporters, whereas short periods of starvation result in derepression of already exisiting transport systems.

In summary, this RNA expression analysis indicates that both transporters indeed may have different physiological functions with respect to phosphate transport in plants. Inhibition of the expression of either or both transporters in transgenic plants will further our understanding of these processes in future.

METHODS

Enzymes and Chemicals

Chemicals were obtained from Sigma and Merck (Darmstadt, Germany). DNA restriction enzymes were obtained from New England Biolabs (Beverly, MA) and Boehringer Mannheim. cDNA synthesis kit λ ZAPII was supplied by Stratagene (La Jolla, CA).

Plants, Bacteria, and Yeast Strains

Strains DH5α (Bethesda Research Laboratories) and XL1 Blue (Stratagene) from *Escherichia coli* were cultured using standard techniques (Sambrook et al., 1989). Yeast strain MB192 (*MAT***a** *pho3-1 pho84::HIS3 ade2 leu2-3,112 his3-532 trp1-289 ura3-1,2 can1*) was kindly provided by S. Harashima and Y. Oshima (Osaka University, Osaka, Japan). Potatoes (*Solanum tuberosum* cv Désirée) were obtained from Saatzucht Fritz Lange KG (Bad Schwartau, Germany). Plants were grown in the greenhouse with a 16-hr-light/8-hr-dark regime at 20 and 15°C, respectively. Plants were grown in sealed pots on 0.5 × Hoagland's medium as described by Röhm and Werner (1987). The medium was changed to tap water for the final 3 days before harvest. Selectively nutrient-deprived plants were grown in quartz sand culture supplied with 0.5 × Hoagland's medium in which the omitted salt was replaced by an appropriate ion such as Cl^-, NO_3^-, Ca^{2+}, Na^+, or K^+.

cDNA Library Synthesis and Screening

To isolate cDNAs that encode phosphate transporters involved in the uptake of phosphate from soil, we established a cDNA library from potato roots. Plants were grown in 0.5 × Hoagland's medium for 5 weeks in sealed pots. Before harvest, the medium was changed to tap water for the final 3 days of growth. cDNA synthesis from poly(A)$^+$ RNA extracted from harvested roots was performed using a cDNA synthesis kit (Stratagene). A cDNA >800 bp long was selected to create a library in the λ ZAP vector. Recombinant independent clones (7×10^5) were obtained, with lengths ranging from 0.8 to 3.5 kb.

Recombinant λ phages (5×10^5) were screened using the SalI and SmaI cDNA insert of expressed sequence tag clone 134M11T7 (kindly provided by the Arabidopsis Biological Research Center, Ohio State University, Columbus). The cDNA insert of this clone is 1 kb, and the 300-bp segment that has been sequenced has 35% identity to *PHO84* when the deduced amino acid sequences are compared. Filters were hybridized overnight at 42°C in polyethylene glycol buffer containing 22% formamide (Amasino, 1986). Filters were washed for 20 min at 42°C in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS. After two rounds of screening, the plaque-purified phages were converted to pBluescriptII SK− derivatives by in vivo excision.

DNA and Peptide Sequence Analysis

DNA sequence analysis was performed using synthetic oligonucleotides in conjunction with T7 polymerase. All other methods were done according to Sambrook et al. (1989). Computer analysis was performed using the software package of the Genetics Computer Group (Madison, WI) and the PROSITE data bank (PROSITE: A dictionary of protein sites and patterns, WWW server: http://expasy.hcuge.ch/). In the given consensus sequences, brackets indicate that either of the given amino acids should be present, traces allow any amino acids at that position except the one given, x indicates that any amino acid is accepted, and x(2) indicates a repetition.

Yeast Transformation

Putative phosphate transporter cDNAs were subcloned into the NotI site of the yeast expression vector p112A1NE (Riesmeier et al., 1992). Competent yeast MB192 cells were prepared and transformed with the construct according to Dohmen et al. (1991).

Acid Phosphatase Activity Test and Yeast Growth Experiments

Yeasts were plated on staining medium and stained for acid phosphatase activity (Bun-ya et al., 1991). Essentially, yeasts were cultured with the appropriate amino acids on YNB medium (Difco), the sucrose of which (20 g/L) was replaced by 30 g/L glycerol. Repressible acid phosphatase activity was monitored by embedding the colonies in top agar, which contained components of a diazo-coupling reaction mix. The mix consisted of 5 mg of α-naphthylphosphate and 50 mg of *o*-dianisidine dissolved in 10 mL of a 0.5% liquid soft agar, which had been prepared with 0.1 M acetate buffer, pH 4.0, and precooled to 50°C. Precipitation of a red dye indicated phosphatase activity. Growth was monitored in liquid YNB medium by optical density measurements at 660 nm.

Uptake of Radioactive Orthophosphate

Cells were grown to the logarithmic phase on modified YNB medium containing only 140 μM KH_2PO_4. Cells were washed in Pi-free medium and resuspended in the same medium to 2.5% (w/v). Uptake was performed as described by Cirillo (1989). Cells (1 mg) were incubated in a solution containing 140 μM Pi together with 100 nCi phosphorus-32. The incorporation reaction was stopped by transfer to 4 mL of ice-cold water and subsequent filtration on glass-fiber filters (Whatman). After two additional washings with 4 mL of ice-cold water, the radioactivity incorporated by the cells was determined using a liquid scintillation counter (Beckman Instruments). The reaction was performed at room temperature, and yeast cells were preincubated with 10 mM glucose for 5 min. For inhibition studies, the reagents were added 30 sec before the addition of the labeled orthophosphate. Inhibitors were dissolved in appropriate solvents and used at a final concentration of 10 and 100 μM of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (2,4-DNP), respectively. Mes buffer at a final concentration of 25 mM was used to determine transport activity at different pH values.

RNA Gel Blot Hybridization

Total RNA from plant material was isolated according to Logemann et al. (1987). After denaturation in 40% formamide and subsequent separation on 1.5% agarose gels containing 15% formaldehyde, the gels were blotted in 20 × SSC onto Hybond N membranes (Amersham International). NotI cDNA inserts of clones p12 and p21 were used to prepare radioactively labeled probes. Hybridization was performed overnight in polyethylene glycol buffer containing 22% formamide (Amasino, 1986) at 42°C. Blots were washed at 42°C in 2 × SSC for 30 min followed by a wash in 0.2 × SSC at 60°C for another 20 min. Blots were exposed between intensifying screens at −70°C for 16 hr to Kodak XAR-5 films.

ACKNOWLEDGMENTS

We thank Yasuji Oshima and Satoshi Harashima (University of Osaka) for yeast strain MB192, the Arabidopsis Biological Resource Center for providing us with the EST.cDNA clone 134M11T7, and Frank W. Smith (Commonwealth Scientific and Industrial Research Organization, Brisbane, Australia) and Richard N. Trethewey for critically reading the manuscript and for helpful discussions.

Received October 16, 1996; accepted January 21, 1997.

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NOTE ADDED IN PROOF

Subsequent to submission of this paper, more members of the plant phosphate transporter family have been reported. Two cDNAs, *AtPT1* and *AtPT2* (**Muchhal, U.S., Pardo, J.M., and Raghothama, K.G.** [1996]. Phosphate transporters from the higher plant *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **93,** 10519–10523), and two genes, *APT1* and *APT2* (**Smith, F.W., Ealing, P.M., Dong, B., and Delhaize, E.** [1997]. The cloning of two *Arabidopsis* genes belonging to a phosphate transporter family. Plant J. **11,** 83–92), have been described.